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## Age-dependent glycosylation of the sodium taurocholate cotransporter polypeptide: From fetal to adult human livers

Sargiacomo, Camillo ; El-Kehdy, Hoda ; Pourcher, Guillaume ; Stieger, Bruno ; Najimi, Mustapha ; Sokal, Etienne

**Abstract:** Sodium taurocholate cotransporter polypeptide (NTCP), mainly expressed on the sinusoidal membrane of hepatocytes, is one of the major transporters responsible for liver bile acid (BA) re-uptake. NTCP transports conjugated BA from the blood into hepatocytes and is crucial for correct enterohepatic circulation. Studies have shown that insufficient hepatic clearance of BA correlates with elevated serum BA in infants younger than 1 year of age. In the current study, we investigated human NTCP messenger RNA and protein expression by using reverse-transcription quantitative polymerase chain reaction and immunoblotting in isolated and cryopreserved human hepatocytes from two different age groups, below and above 1 year of age. Here, we show that NTCP messenger RNA expression is not modulated whereas NTCP protein posttranslational glycosylation is modulated in an age-dependent manner. These results were confirmed by quantification analysis of NTCP 55-kDa N-glycosylated bands, which showed significantly less total NTCP protein in donors below 1 year of age compared to donors older than 1 year. NTCP tissue localization was also analyzed by means of immunofluorescence. This revealed that NTCP cellular localization in fetal samples was mainly perinuclear, suggesting that NTCP is not glycosylated, while its postnatal localization on the plasma membrane is age dependent compared to multidrug resistant protein 2, which is apical starting in fetal life. Conclusion: After birth, the NTCP age-dependent maturation process requires approximately 1 year to complete NTCP glycosylation in human hepatocytes. Therefore, NTCP late posttranslational glycosylation appears to be important for correct NTCP membrane localization, which might explain physiologic cholestasis in neonatal life and might play a central role for HBV infection after birth. (Hepatology Communications 2018;2:693-702)

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# Age-Dependent Glycosylation of the Sodium Taurocholate Cotransporter Polypeptide: From Fetal to Adult Human Livers

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Sodium taurocholate cotransporter polypeptide (NTCP), mainly expressed on the sinusoidal membrane of hepatocytes, is one of the major transporters responsible for liver bile acid (BA) re-uptake. NTCP transports conjugated BA from the blood into hepatocytes and is crucial for correct enterohepatic circulation. Studies have shown that insufficient hepatic clearance of BA correlates with elevated serum BA in infants younger than 1 year of age. In the current study, we investigated human NTCP messenger RNA and protein expression by using reverse-transcription quantitative polymerase chain reaction and immunoblotting in isolated and cryopreserved human hepatocytes from two different age groups, below and above 1 year of age. Here, we show that NTCP messenger RNA expression is not modulated whereas NTCP protein posttranslational glycosylation is modulated in an age-dependent manner. These results were confirmed by quantification analysis of NTCP 55-kDa N-glycosylated bands, which showed significantly less total NTCP protein in donors below 1 year of age compared to donors older than 1 year. NTCP tissue localization was also analyzed by means of immunofluorescence. This revealed that NTCP cellular localization in fetal samples was mainly perinuclear, suggesting that NTCP is not glycosylated, while its post-natal localization on the plasma membrane is age dependent compared to multidrug resistant protein 2, which is apical starting in fetal life. **Conclusion:** After birth, the NTCP age-dependent maturation process requires approximately 1 year to complete NTCP glycosylation in human hepatocytes. Therefore, NTCP late posttranslational glycosylation appears to be important for correct NTCP membrane localization, which might explain physiologic cholestasis in neonatal life and might play a central role for HBV infection after birth. (*Hepatology Communications* 2018;2:693-702)

The liver is responsible for the transport of solutes from the portal venous circulation to the canaliculus by bile acid (BA) vectorial transport. Solute carrier transporters are crucial for an active vectorial BA uptake.<sup>(1,2)</sup> Among these, the sodium taurocholate cotransporter polypeptide (NTCP; SLC10A1) is a major BA uptake transporter in the liver that preferentially moves conjugated BA into mature hepatocytes in a sodium-dependent manner.<sup>(3-5)</sup> In humans, NTCP accounts for more than 80% of the hepatic uptake of bile salts (Na<sup>+</sup>-dependent pathway).<sup>(6-8)</sup> Alternatively, isoforms of the organic anion

*Abbreviations:* AFP, alpha-fetoprotein; BA, bile acid; CYP, cytochrome P450; mRNA, messenger RNA; MRP2, multidrug resistance protein 2; NTCP, sodium taurocholate cotransporter polypeptide; PHH, primary human hepatocyte; PNGase F, peptide-N4-(N-acetyl-beta-glucosaminyl); qPCR, quantitative polymerase chain reaction; RIPA, radio immunoprecipitation assay; SDS, sodium dodecyl sulfate;  $\alpha$ -SMA, alpha smooth muscle actin.

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Potential conflict of interest: Dr. Najimi and Dr. Sokal advise and own stock in Promethera. The other authors have nothing to report.

transporting polypeptides multispecific transporter family compensate for NTCP deficiency by internalizing conjugated and unconjugated BA ( $\text{Na}^+$ -independent pathway).<sup>(9,10)</sup> Patients with primary hypercholanemia have been described with relatively mild clinical phenotypes, which have also been associated with growth retardation and vitamin deficiency.<sup>(11,12)</sup> Interestingly, down-regulation of plasma membrane NTCP was reported in cases of progressive familial intrahepatic cholestasis-2 and -3<sup>(13)</sup> and was thought to be linked to NTCP posttranslational modifications.

Structurally speaking, NTCP is a 55-kDa N-glycosylated multipass transmembrane protein composed of 349 amino acids that shares over 70% sequence similarity with rat Ntcp.<sup>(14,15)</sup> On the N-terminal domain, two N-linked glycosylation sites (Asn 5, Asn 11) facing the extracellular lumen have been characterized<sup>(15,16)</sup> and are important for NTCP localization on the plasma membrane.<sup>(17)</sup> Current knowledge of NTCP ontogenesis is mostly based on experiments conducted on rats and shows that i) compared to adult levels, Ntcp messenger RNA (mRNA) levels at birth are 35%-76%; ii) in newborns, Ntcp protein expression reaches adult levels during the first postnatal week; iii) Ntcp expression levels and plasma membrane localization do not correlate with protein functional status; iv) NTCP activity is delayed approximately 4 weeks after birth.<sup>(6,18-21)</sup> However, extrapolations in humans are limited due to the known differences among species in NTCP expression regulation. In this work, NTCP gene and protein expression in fetal, neonatal, infant, and adult livers were investigated to better understand human NTCP ontogeny. Here, we found by immunoblotting analysis that the NTCP glycosylation process is completed after approximately 1 year of age. We measured NTCP 55 kDa total protein amounts between hepatocyte samples above and below 1 year of age and found that donors younger than 1 year

expressed significantly lower amounts of mature NTCP protein compared to donors older than 1 year and adults. Then, we again show by immunofluorescence analysis that NTCP protein is gradually expressed on the plasma membrane in an age-dependent manner and is absent on the hepatocyte surface of fetal livers. Overall, these findings show for the first time that NTCP N-glycosylation requires several months to completely functionally localize on the plasma membrane of mature hepatocytes.

## Materials and Methods

### CRYOPRESERVED PRIMARY HUMAN HEPATOCYTES

Twelve isolated hepatocyte specimens from donors with no known NTCP-related pathology were obtained from the human liver and liver stem cell tissue bank, Center for Tissular and Cellular Therapy, Cliniques Universitaires St. Luc (Brussels, Belgium). Consent to use the tissue for research was obtained from the legal guardians (Table 1). Human fetal livers were carefully dissected from fetuses collected after voluntary pregnancy termination at gestational weeks 12-13 with the mothers' written consent, abiding by the French ethical guidelines and the amended Declaration of Helsinki.

Hepatocyte isolation was performed by using two-step collagenase perfusion under good manufacturing practice-like conditions.<sup>(22-25)</sup> The isolated hepatocytes were stored in liquid nitrogen until use. Batches of cryopreserved hepatocytes were rapidly thawed in a 37°C water bath and washed twice using thawing solution. After two centrifugations at 400g for 5 minutes at 4°C, hepatocyte pellets of approximately 5 million and 50 million cells were suspended in lysis buffer specific for RNA or protein analysis.

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**TABLE 1. CHARACTERISTICS OF THE TWO LIVER DONOR GROUPS USED IN THE CURRENT STUDY FOR IMMUNOBLOTTING**

Donor Above 1 Year of Age	Age	Sex
1	2 years old	F
2	3 years old	F
3	3 years old	M
4	4 years old	F
5	14 years old	M
6	26 years old	F
Donor Below 1 Year of Age	Age	Sex
7	3 days old	M
8	6 days old	F
9	1 month old	M
10	4 months old	M
11	7 months old	F
12	1 year old	M

## NTCP mRNA EXPRESSION ANALYSIS

Total RNA extraction was performed by dissolving cell pellets with Tripure reagent (Roche, Belgium). Approximately 2  $\mu$ g of total RNA were reverse transcribed by the High Capacity complementary DNA (cDNA) Reverse Transcription Kit (Life Technologies, Belgium). For each quantitative polymerase chain reaction (qPCR), a total of 25 ng input cDNA was analyzed in duplicate by TaqMan gene expression assays (Table 2).  $\Delta\Delta$ Ct comparative analysis was used to evaluate the expression of genes encoding multidrug resistance protein 2 (MRP2), cytochrome P450 3A4 (CYP3A4), CYP7A1, alpha-fetoprotein (AFP), alpha smooth muscle actin ( $\alpha$ -SMA), and by using the peptidylprolyl isomerase A housekeeping gene as control. Primary human hepatocytes (PHHs) from a 3-day-old donor were used as the reference sample. Results are expressed for all tested genes as mRNA increase (expressed as fold increase compared to reference sample [Donor 7, 3 days old]).

## WESTERN BLOTTING

Total protein lysates were obtained by dissolving cell pellets from 5 million–10 million PHHs in radio immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris base, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with a cocktail of protein inhibitors (Roche). Samples were then vortexed, left on ice for a few minutes, sonicated with an Ultrasonicator homogenizer (Sonopuls) for at least 30 seconds, and incubated for an additional 30 minutes at 4°C. Clarification was performed by centrifugation at maximum

speed (20,800 rcf) for 15 minutes. Pellets were resuspended in RIPA buffer for protein quantification analysis. Fifty micrograms of PHH samples were boiled in loading buffer (100 mM Tris-HCl [pH 6.8], glycerol 4% [volume {vol.}/vol.], SDS [weight/vol.], dithiothreitol 200 mM, bromophenol blue 0.2%) at 95°C for 5 minutes. Protein gel separation was performed using a 10% Tris-glycine SDS-polyacrylamide gel electrophoresis gel that was then blotted overnight onto polyvinylidene fluoride membranes. Membranes were then blocked for 1 hour using 5% bovine serum albumin blocking solution and subsequently incubated overnight at 4°C in blocking solution containing rabbit anti-NTCP (1:1,000).<sup>(26)</sup> Fluorescent-labeled secondary antibodies (Biotium) were then used for 40 minutes at room temperature. Revelation was accomplished by the Odyssey imaging system (LI-COR). Image Studio Light 4 software (LI-COR) was used for quantification analysis.

## PNGase DEGLYCOSYLATION ASSAY

Peptide-N4-(N-acetyl-beta-glucosaminyl) (PNGase F) asparagine amidase de-glycosylation treatment (New England Biolabs) was performed according to the manufacturer's instructions with slight modifications. PHH total protein extracts were treated with PNGase F containing RIPA buffer for 2 hours at 38°C.

## IMMUNOFLUORESCENCE ASSAY

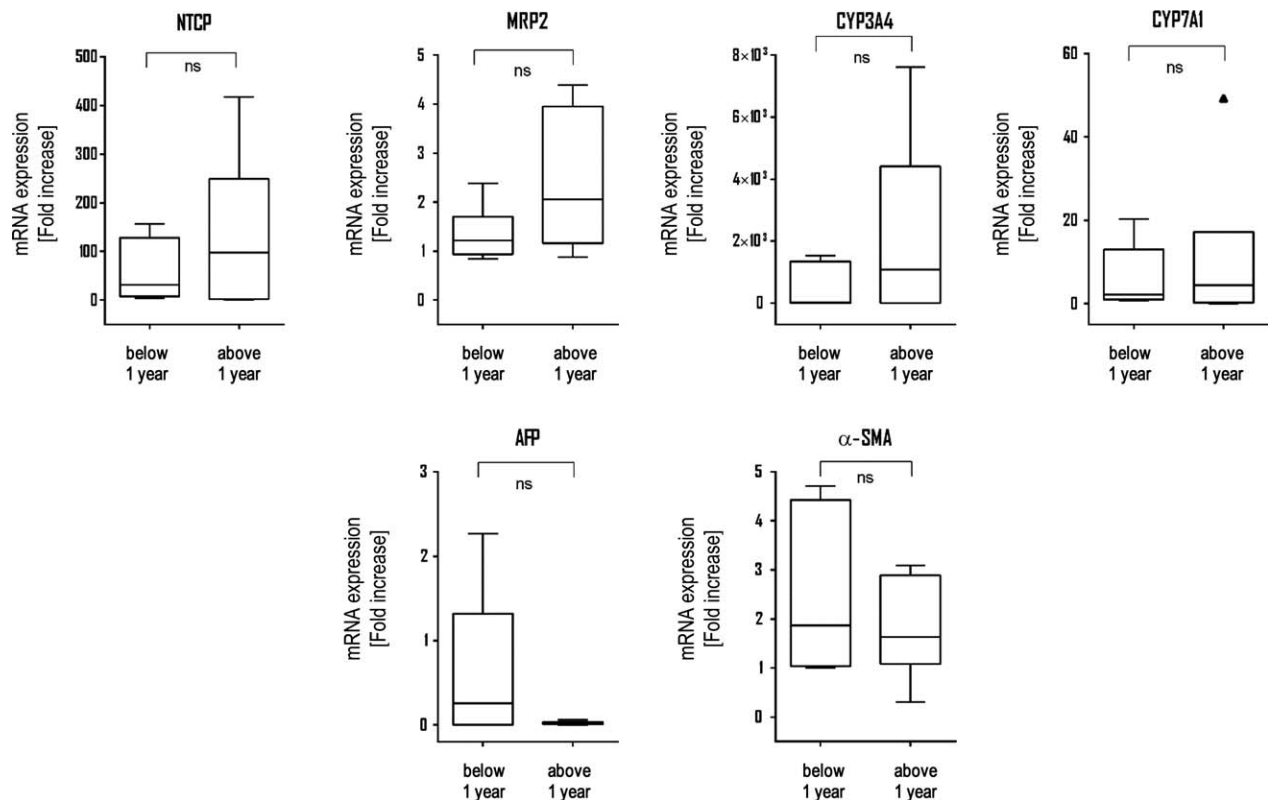
Five micrometer-thick human liver sections (paraffin embedded) at different age stages were deparaffinized and rehydrated in graded alcohol. For antigen retrieval, liver sections were incubated in citric acid monohydrate buffer (Dako), then microwaved for 10 minutes at 720 W. Nonspecific staining was prevented by incubating the slices in 10% bovine serum albumin for 45 minutes at room temperature. Slices were incubated with anti-NTCP at 1:100 (HP A042727; Sigma) and anti-MRP2 at 1:50 (ALX-801-016-C250;

**TABLE 2. TAQMAN PROBES USED FOR GENE EXPRESSION ANALYSIS**

Gene	Reference	Supplier
NTCP	Hs00161820_m1	ThermoFisher
MRP2	Hs00960489_m1	ThermoFisher
CYP3A4	Hs00604506_m1	ThermoFisher
CYP7A1	Hs00167982_m1	ThermoFisher
$\alpha$ -SMA	Hs00559403_m1	ThermoFisher
AFP	Hs01040598_m1	ThermoFisher
PPIA	Hs99999904_m1	ThermoFisher

Abbreviation: PPIA, peptidylprolyl isomerase A.





**FIG. 1.** Hepatocyte relative gene expression analysis. Gene expression analysis of hepatocytes below and above one year of age: NTCP ( $31 \pm 26.0$  versus  $97 \pm 63.7$ ), MRP2 ( $1.3 \pm 0.23$  versus  $2.4 \pm 0.6$ ), CYP3A4 ( $474.6 \pm 296.0$  versus  $2,184 \pm 1,195$ ), and CYP7A1 ( $6.1 \pm 7.8$  versus  $10.8 \pm 19.0$ ); relative gene expression did not significantly increase with age. The expression of genes encoding nonhepatic markers AFP ( $0.6 \pm 0.8$  versus  $0.02 \pm 0.02$ ) and  $\alpha$ -SMA ( $2.470 \pm 1.638$  versus  $1.803 \pm 1.020$ ) was nonsignificantly decreased in donors above 1 year of age. Intravariability was elevated in both groups. Values are expressed as mRNA fold increase versus controls (3-day-old donor) and represent median  $\pm$  SEM. Peptidylprolyl isomerase A was used as the housekeeping reference gene, and hepatocytes from the youngest donor were used as the reference sample. Statistical analysis was performed by the one-tail Mann-Whitney test ( $P > 0.1$ ). Median values are represented with a black horizontal line in each box plot. Abbreviation: ns, not significant.

Enzo) primary antibodies overnight at 4°C. After the washing step, slices were incubated for 1 hour with donkey anti-rabbit at 1:500 (A10042; Invitrogen) and then for one hour with donkey anti-mouse at 1:500 (A31571; Invitrogen) secondary antibodies at room temperature. Nuclei were stained for 5 minutes with 4',6-diamidino-2-phenylindole (Life Technologies). Slides were mounted in Fluoromount medium (Dako). Fluorescence was assessed using an Imager A1 fluorescence microscope (Carl Zeiss), and digital images were acquired using Axiovision software.

## STATISTICAL ANALYSIS

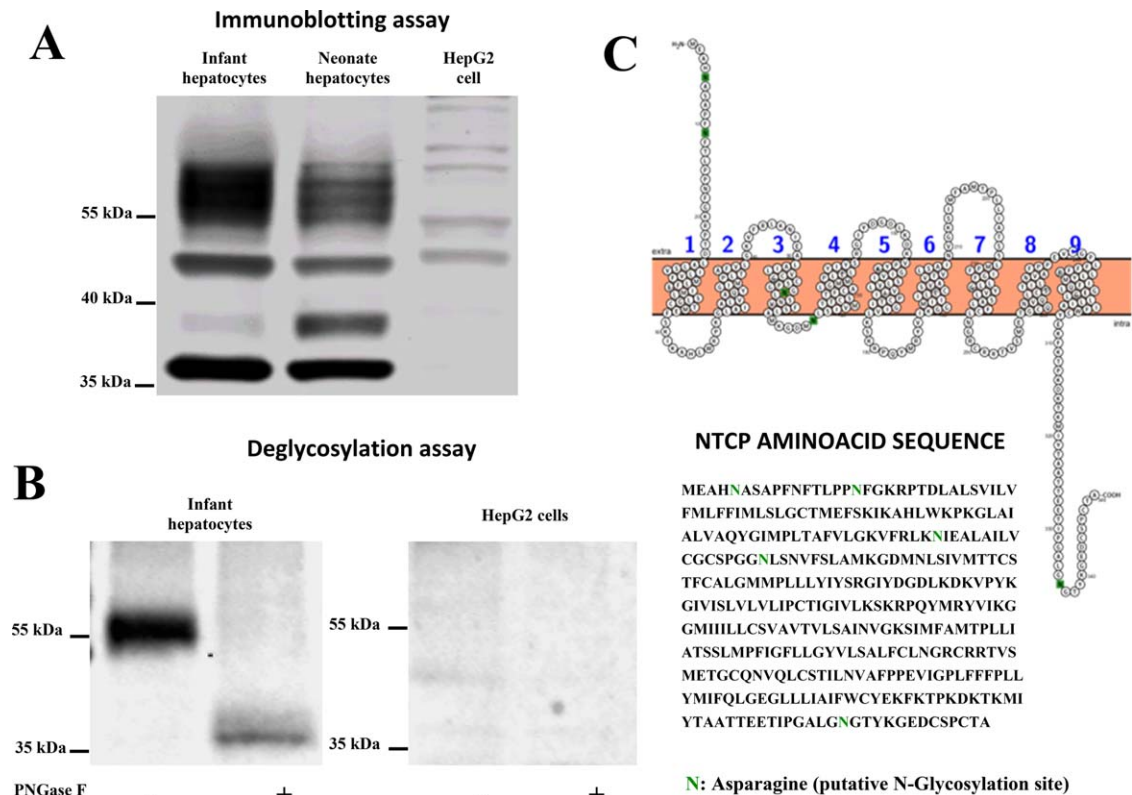
Statistical analysis was performed using GraphPad PRISM 6 software. The Student *t* test and Mann-Whitney U test were used.  $P < 0.01$  was considered

significant. All values are expressed as mean  $\pm$  SEM or median.

## Results

### NTCP mRNA LEVELS ARE NOT DIFFERENTIALLY MODULATED BELOW AND ABOVE 1 YEAR OF AGE

NTCP age-dependent transcriptional modulation was analyzed by measuring the expression of the NTCP gene together with a series of liver-specific and control genes in hepatocyte donors above and below 1 year of age by reverse-transcription qPCR (Fig. 1). Our results showed that expression of typical hepatic



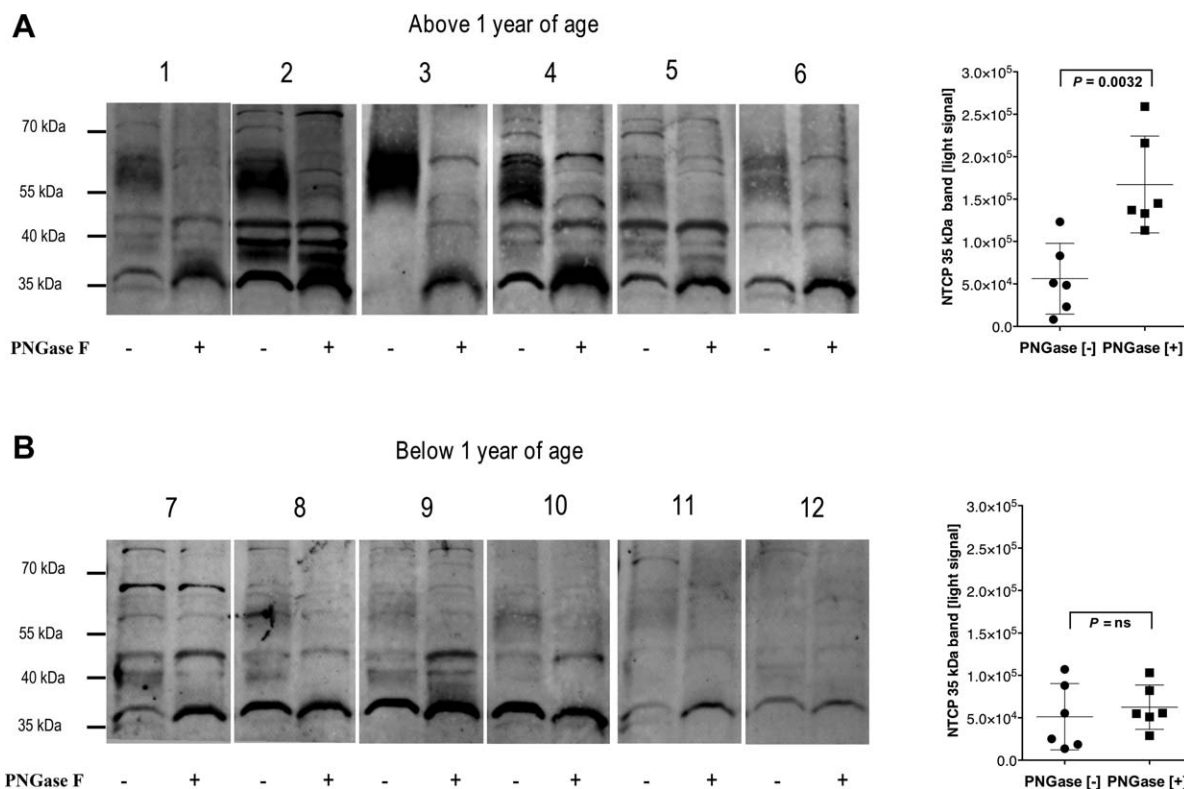
**FIG. 2.** NTCP immunoblotting and N-glycosylation in hepatocytes. (A) Fifty micrograms of hepatocyte total protein extracts from infant (3 years) and neonate (6 days) donors were compared with HepG2 cells by immunoblotting analysis. Anti-NTCP antibody was able to detect multiple NTCP bands (complex glycosylation 55-kDa and nonglycosylated 35-kDa bands). HepG2 cells did not express NTCP protein. (B) Immunoblotting assay showing NTCP deglycosylation by PNGase F between hepatocytes and HepG2 cells. NTCP complex glycosylation 55 kDa was validated for its N-glycosylation sites. (C) NTCP amino acid structure and transmembrane domains in the plasma membrane (UniProt sequence, Q14973). Depicted are five asparagine residues (green circles), two of which, Asp 5 and 11, are N-glycosylated.

markers MRP2, CYP3A4, and CYP7A1 or other fetal AFP and mesenchymal  $\alpha$ -SMA genes were not significantly different between the two groups, mainly due to the intravariability in the mRNA expression levels between donors of the same group. However, compared to donors less than 1 year old, the median values of NTCP (younger than 1 year,  $31 \pm 26.07$  versus older than 1 year,  $97 \pm 63.71$ ), MRP2 ( $1.3 \pm 0.2$  versus  $2.4 \pm 0.5$ ), and CYP3A4 ( $474.6 \pm 296.0$  versus  $2,184 \pm 1,195$ ) data suggested a trend toward enhanced gene expression in hepatocytes above 1 year of age. In contrast, relative gene expression of AFP ( $0.63 \pm 0.89$  versus  $0.02 \pm 0.02$ ) and  $\alpha$ -SMA ( $2.47 \pm 1.63$  versus  $1.80 \pm 1.02$ ) showed, as expected, to be strongly decreased in hepatocytes older than 1 year compared to those younger than 1 year. Remarkably, expression during development of the CYP7A1 hepatic marker for bile salt synthesis was virtually identical ( $6.15 \pm 7.84$  versus  $10.83 \pm 19.04$ ) in

hepatocytes younger and older than 1 year, which suggests an early onset of bile salt synthesis.

## NTCP 55 kDa GLYCOSYLATED BAND IS MODULATED BY AGE

We next investigated NTCP protein expression by western blotting analysis. We first validated the NTCP primary antibody and confirmed that, compared to a negative control of HepG2 cells, hepatocytes from 2 donors, aged 3 years and 6 days, expressed NTCP (Fig. 2A). Glycosylated and nonglycosylated NTCP forms resulted in multiple bands ranging from 35 to 65 kDa that were detected in hepatocytes samples but not in HepG2 cells. Intriguingly, these results indicate that while 35-kDa nonglycosylated NTCP bands were stably expressed in both samples, 55-kDa glycosylated NTCP bands increased in aged donors (Supporting Fig. S1). Moreover, compared with



**FIG. 3.** NTCP immunoblotting and total protein quantification analysis. NTCP immunoblotting analysis of treated and untreated hepatocyte samples with PNGase. NTCP complex glycosylated 55-kDa bands were efficiently digested and resulted in an almost complete band shift of the NTCP signal to a single band at 35 kDa (NTCP total protein) that was quantified in neonate and infant donors below 1 year of age and infants and adults above 1 year of age. (A) NTCP total protein was significantly increased in treated donors above 1 year of age compared to untreated donors ( $P = 0.0032$ ). (B) In contrast, treated and untreated hepatocyte samples of donors less than 1 year of age showed no differences ( $P = 0.5$ ), consistent with poor NTCP glycosylation in infants. Values are expressed as NTCP 35 kDa net signal. Statistical unpaired  $t$  test (two-tailed) was performed ( $P < 0.01$ ). Results are expressed as net signal. Horizontal black lines are the mean of the quantified amount. Abbreviation: ns, not significant.

untreated samples, NTCP 55-65-kDa N-glycosylated bands were effectively deglycosylated with PNGase F and produced a band shift to 35 kDa (Fig. 2A; Supporting Fig. S2). HepG2 cells did not express NTCP, and PNGase digestion did not produce any molecular weight shift in the NTCP bands.

### COMPLETION OF NTCP GLYCOSYLATION REQUIRES AT LEAST 1 YEAR

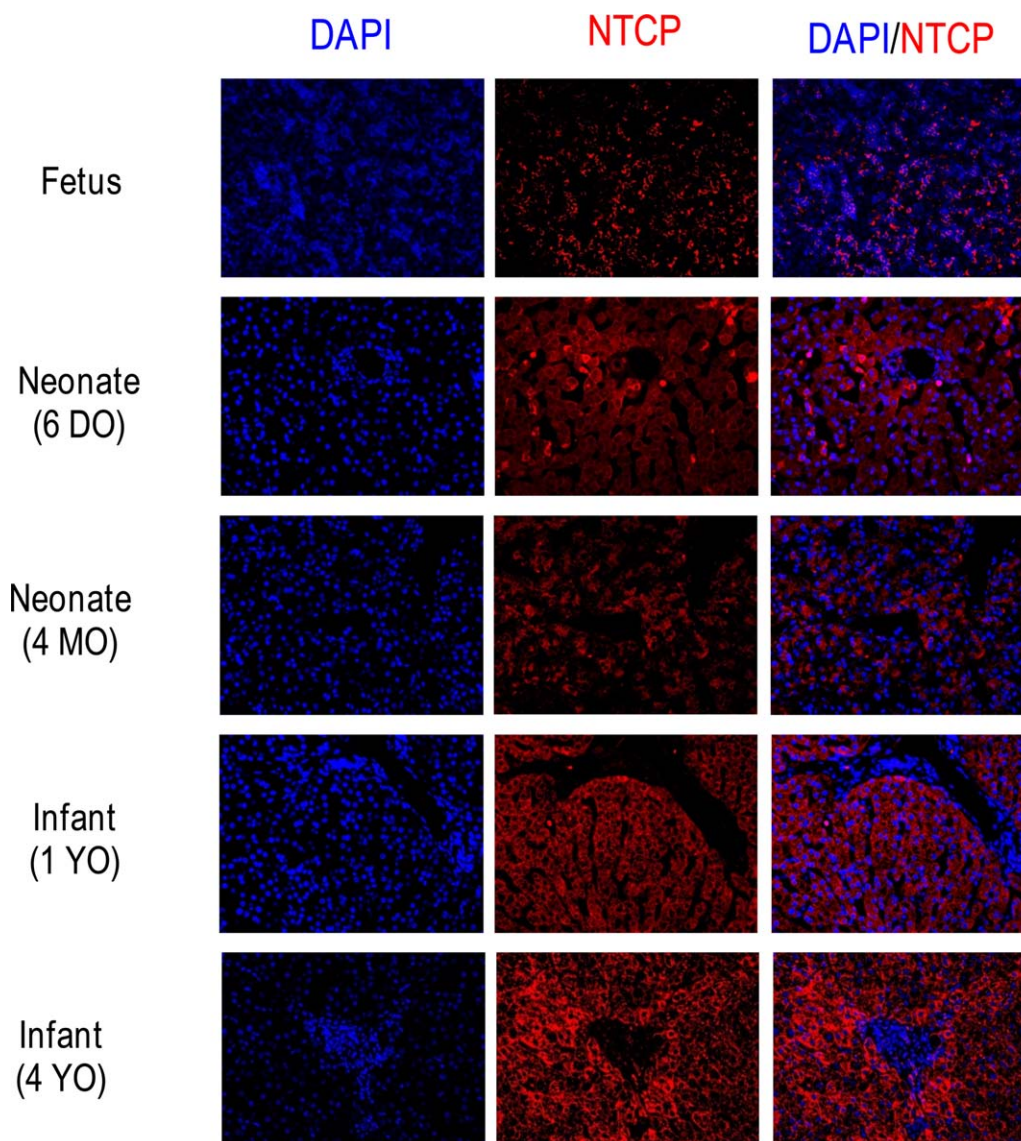
Subsequently, we analyzed hepatocyte samples from 6 donors less than 1 year and 6 donors older than 1 year by immunoblotting and densitometry (Fig. 3). Compared to donors below 1 year, NTCP 55-65-kDa bands were highly expressed in samples above 1 year and were confirmed by PNGase treatment compared

to nontreated samples ( $P = 0.0032$ ) (Fig. 3). In contrast, we found that NTCP glycosylation of hepatocytes below 1 year was poor and that compared to untreated samples, PNGase F treatment failed to increase NTCP total protein ( $P = 0.5$ ). We also observed that NTCP nonglycosylated protein pools (untreated lanes) were comparable between donors below and above 1 year of age, suggesting a stable quantity of the native NTCP protein pool.

### NTCP AND MRP2 ARE DIFFERENTIALLY REGULATED DURING LIVER DEVELOPMENT

Human liver sections from fetal donors and infants older and younger than 1 year were analyzed by immunofluorescence to study NTCP localization and to

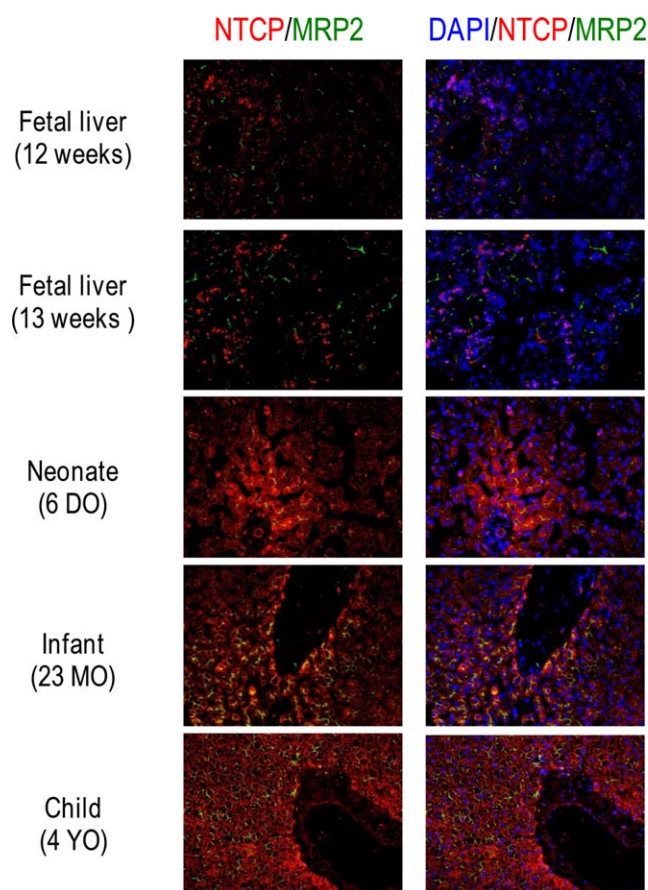




**FIG. 4.** NTCP immunofluorescence in fetal, neonate, and infant liver tissues. Liver tissue samples were analyzed by immunofluorescence ( $n = 5$ ). Each row represents 1 donor. Columns starting from left show nuclear staining using DAPI (blue), NTCP staining (red), and merged staining (blue/red). In fetal livers, NTCP localization was mainly intracytoplasmic. Compared to prenatal samples, 6-day-old and 4-month-old donors in postnatal condition showed increased NTCP expression; NTCP plasma localization at 1 and 4 years of age is almost exclusively found on the plasma membrane (magnification  $\times 200$ ). Abbreviations: DO, days old; MO, months old; DAPI, 4',6-diamidino-2-phenylindole; YO, years old.

investigate if the lack of glycosylation revealed by immunoblotting correlates with an effective reduction of NTCP membranous expression between prenatal and postnatal stages ( $n = 5$ ) (Fig. 4). We found that postnatally, NTCP protein expression was relatively low and, when detected, was mainly localized in the cytoplasm, as observed in liver samples below 1 year of age. However, compared to mature livers where NTCP is found on the plasma membrane, less

immunostaining was detected in a 4-year-old donor. Indeed, to our knowledge, this is the first time that NTCP localization has been reported to be found mainly in the cell cytoplasm of fetal hepatocytes (Fig. 5). In contrast, MRP2 localization in fetal hepatocytes appeared with a sharp linear canalicular staining similar to mature liver but with a more linear canalicular structure (Fig. 6); no cytoplasm localization was observed during development, indicating that NTCP and MRP2



**FIG. 5.** NTCP and MRP2 distribution in human fetal and infant liver tissues. Liver tissues co-immunostained with NTCP (red) and MRP2 (green) were analyzed in fetal ( $n = 2$ ) and postnatal ( $n = 3$ ) livers and counterstained with DAPI. NTCP localization in fetal and neonatal hepatocytes was found in the cytoplasm; NTCP localization becomes more expressed on the surface of hepatocytes with age. In contrast, MRP2 distribution was found to be expressed apically and was not found in the cytoplasm (magnification  $\times 200$ ). Abbreviations: DO, days old; MO, months old; DAPI, 4',6-diamidino-2-phenylindole; YO, years old.

are differentially regulated at different developmental stages.

## Discussion

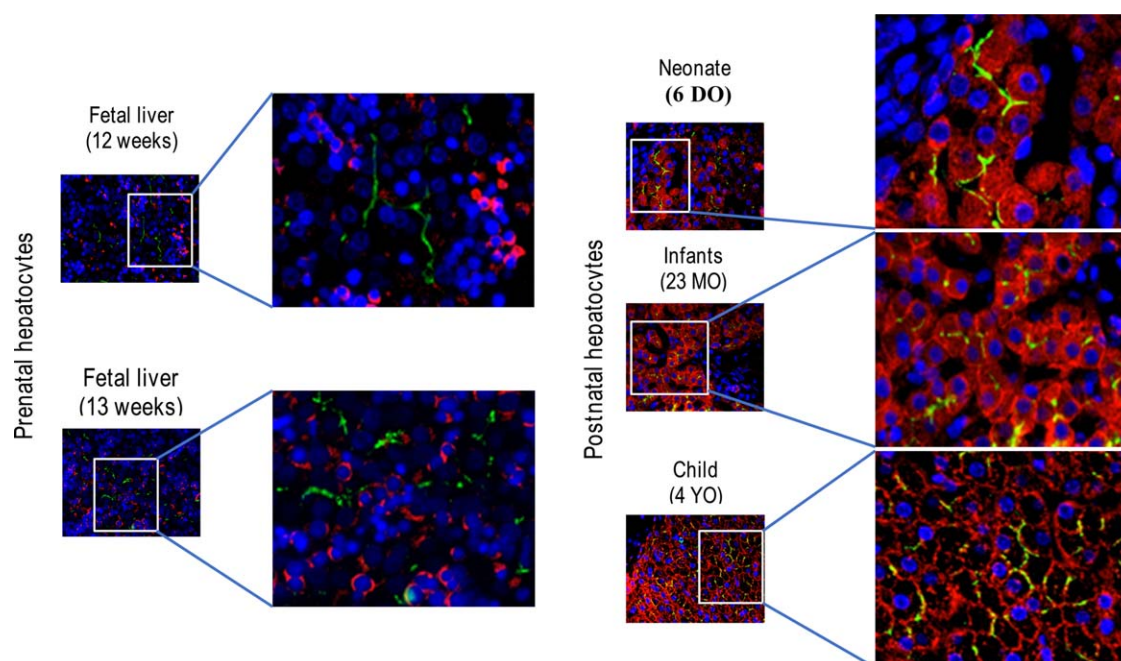
Immediately following birth, numerous and dramatic changes occur in hepatic blood flow and in the structural and functional organization of the liver.<sup>(27)</sup> Our study suggests that NTCP protein N-glycosylation is age dependent and is not completed until approximately 1 year after birth. In contrast, Ntcp glycosylation in rats was shown to be completed within

4 weeks after birth, illustrating significant species differences in transporter expression, as described.<sup>(28,29)</sup> Additionally, by using immunofluorescence, our study revealed that the adult NTCP membrane pattern was reached only after 1 year of age and was strongly correlated with the net increase of the NTCP 55-kDa complex glycosylated band previously detected by immunoblotting analysis in hepatocytes older than 1 year. In contrast, MRP2 is already located at the apical membrane of hepatocytes in fetal and neonatal liver samples, indicating different regulation mechanisms for these transporters during development.

Our findings are also supported by clinical data suggesting that “physiologic cholestasis” is caused by immature posttranslational glycosylation modifications of NTCP.<sup>(30,31)</sup> Under physiological conditions, the fetus presents a low serum BA pool (0.1 mM) that increases postnatally (1–2 mM). These drastic changes after birth indicate that BA clearance compensatory activity by the placenta is lost in the fetus and that liver clearance functions are insufficient in most neonates.<sup>(28,32)</sup> Clinical evidence shows in most cases that serum BA levels reach adult values (0.5 mM) only after 1 year of age, again supporting the increase in NTCP glycosylation demonstrated in our study (Figs. 3 and 6). In general, BA concentration levels in plasma are an indirect marker for cholestasis in neonates. It was previously shown in infants that total conjugated chenodeoxycholate blood levels are elevated before 1 year of age, thus suggesting that hepatic clearance of BAs is progressively enhanced during human development by increasing the number of membranous hepatobiliary transporters.<sup>(33)</sup> A more recent study using high-performance liquid chromatography analysis demonstrated that total serum bile salt levels in the blood before 1 year of age are 90% higher than after 1 year of age.<sup>(34)</sup> Overall, these studies are consistent with our findings suggesting that incomplete NTCP glycosylation might be responsible for hypercholanemia in early infancy.

Furthermore, we found that NTCP protein was mainly localized in the cytoplasm of fetal livers and was absent from the surface of immature hepatocytes (Fig. 5), which suggests a key compensatory BA uptake activity by the placenta BA transporters. We also show in Fig. 6 that MRP2 distribution in neonates and infants is exclusively localized at the apical membrane and is absent from the cytoplasm, which is in line with other studies conducted on human fetal liver samples.<sup>(35)</sup> These results indicate a different regulation between MRP2 and NTCP protein expression and confirm that the hepatocyte maturation process





**FIG. 6.** NTCP and MRP2 localization in human hepatocytes. Immunostaining of NTCP basolateral marker (red), MRP2 apical marker (green), and DAPI nuclear staining (blue) in hepatocytes from a neonate to infants (left figure magnification  $\times 200$ ). Figures on the right hand are scaled images of the 200x. Abbreviations: DO, days old; MO, months old; DAPI, 4',6'-diamidino-2-phenylindole; YO, years old.

toward a fully polarized transporter phenotype is a late event with respect to NTCP.

Transporter glycosylation is differentially regulated by the liver–gut axis pathway, i.e., BA, cortisol, and other soluble agents from the diet or secreted by gut ileocytes.<sup>(9)</sup> *In vitro* studies have shown that dexamethasone increases NTCP mRNA and protein expression by glucocorticoid receptor activation.<sup>(36,37)</sup> Therefore, it would be interesting to investigate the impact of dexamethasone on NTCP glycosylation, particularly after NTCP functional characterization as the first hepatitis B virus receptor.<sup>(38)</sup> In addition to glycosylation, other NTCP posttranslational modifications might participate in modulating NTCP subcellular localization during development. In particular, phosphorylation/dephosphorylation, a cyclic adenosine monophosphate-dependent process, regulates NTCP localization in mature hepatocytes.<sup>(1,39)</sup> It would now be interesting to investigate how phosphorylation/dephosphorylation regulates NTCP localization in fetal hepatocytes. In conclusion, NTCP glycosylation is completed within 1 year after birth and could be used as a potential clinical diagnostic marker for neonates suffering from high BA plasma levels and studied in the context of HBV infection.

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## Supporting Information

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